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THE ROLE OF EXOENZYME S IN PSEUDOMONAS AERUGINOSA INFECTIONS

FINAL REPORT

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We utilized genetic and immunological methods to determine the role of exoenzyme S in Pseudomonas aeruginosa infections. An exoenzyme S deficient mutant, 388 exsl:Tnl, was compared to its S+ parent strain in the burned mouse model and neutropenic rat model. In both animal models the S- mutant was less virulent than its S+ parent strain. The S- mutant was, however, able to establish at the site of infection but it was unable to disseminate. We also developed an ELISA assay to quantitate S antibodies. We determined the ELISA titer and neutralization titer in serum from patients with P. aeruginosa

BLOCK 20 Abstract (continued):

infections, patients with acute infections due to other bacterial species and from healthy control individuals. Healthy individuals or patients infected with species other than P. aeruginosa had either no, or very low, antibody to exoenzyme S. Patients infected with exoenzyme S producing strains developed high titers of S antibody within 3 weeks. These data indicate that exoenzyme S is produced in vivo in humans. We also cloned and partially characterized an exoenzyme S gene from a Pseudomonas aeruginosa chromosomal DNA. The S gene was subcloned and localized to a 3Kb region as one end of an 8.2 Kb DNA fragment. We obtained the sequence for the first 22 amino acids of the amino terminus of the purified 49Kd exoenzyme S protein. Keywords:

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Summary

A.   The long term goal of this research was to determine the role of exoenzyme S in *Pseudomonas aeruginosa* infections. The period covered by this progress report is June 1, 1985 through October 31, 1986. The progress we made is summarized below.

1.   We explored the role of exoenzyme S in the pathogenesis of *P. aeruginosa* infections. We extended our preliminary experiments comparing the ability of the exoenzyme S deficient mutant 388 exs1::Tn1 and its S^+ parent strain to establish and grow in the burned mouse skin at the site of infection and to disseminate. Both the S^- mutant the S^+ parent strain were able to establish at the site of infection and both grew to equally high density. By 24 hrs. after infection, the S^+ parent strain was recovered from the blood or liver and its numbers continued to increase through 48 hrs. In contrast, far fewer S^- mutant bacteria were recovered from the blood or liver. When animals were infected with equal mixtures of the S^+ parent and S^- mutant, the S^- mutant was then able to disseminate well. These results indicated that exoenzyme S was not required for colonization but that it enhanced dissemination of *P. aeruginosa*. These results were strengthened using antibody to purified S. Treatment of mice with anti-S antibody greatly reduced dissemination of the exoenzyme S^+ producing parent strain but did not prevent colonization of the burned skin. The data suggested that exoenzyme S may alter host defenses. (Nicas, T.I., Bradley, J., Lochner, J.E. and Iglewski, B.H., J. Infect. Dis. 152:716, 1985, copies enclosed.)

2.   Studies on the contribution of exoenzyme S to virulence were extended using a neutropenic rat model in collaboration with Dr. Alan Cross, Dr. Jerald Sadoff and Hugh Collins at WRAIR. Two different routes of infection were used; oral and subcutaneous. In both cases, the exoenzyme S deficient mutant (388-exs1::Tn1) was much less virulent (at least 2 logs) than the S^+ parent or the Tn1 induced S^+, leucine auxotroph. Again, the S deficient mutant was able to colonize but was unable to disseminate. These studies were important because they provided the first evidence that any extracellular product produced by *P. aeruginosa* contributed to infections in neutropenic animals and they suggested that exoenzyme S may adversely affect some aspect of host defenses other than neutrophils.

3.   We developed an ELISA assay to quantitate S antibodies. We determined the ELISA titer and neutralization titer in serum from patients with *P. aeruginosa* infections, patients with acute infections due to other bacterial species, and from healthy control patients. First, the ELISA titers correlated very well to the enzyme neutralization titers. Second, healthy individuals or patients infected with species other than *P. aeruginosa* had either no/or low antibody to S. Finally, serial serum samples from several patients who initially had no detectable S antibody (either in ELISA or neutralizing assay) developed high titers of S antibody within 3 weeks. This study indicated that S was produced *in vivo* and that patients developed antibody to S. It also demonstrated the reliability of the ELISA assay for any additional larger study

required to determine if S antibody correlates with recovery from *P. aeruginosa* infections.

    4.   We isolated and began to characterize monoclonal antibodies specific for exoenzyme S. From 4 separate fusions, a total of 8 stable clones were isolated and recloned. All 8 monoclonal antibodies reacted with S in Western blots, and 5 neutralized S enzyme activity.

    5.   We located an exoenzyme S gene on a 28kb piece of DNA inserted into the cosmid, pLAFR, giving us the plasmid pDF100. This was subcloned down to 8.2kb in pLAFR giving the plasmid pDF102. The S gene was not expressed in *E. coli* but was expressed in *P. aeruginosa*. We mutagenized pDF102 with the transposon Tn501 and analyzed these mutants to determine if the Tn501 insertion inactivated the S gene. Thirteen of the mutant plasmids (11 were S$^-$ and 2 S$^+$) were purified and analyzed by restriction mapping. The S gene was localized to a 3kb region at one end of the 8.2kb DNA fragment.

    6.   We determined the sequence for the first 22 amino acids of the amino terminus of the purified exoenzyme S protein. The purified, 49,000 dalton, enzymatically active form of the protein was used for this analysis. We attempted to sequence the 53,000 dalton form of exoenzyme S but it was not able to be sequenced suggesting that its amino terminus was blocked.

B.   Publications during period covered by this report.

    1.   Nicas, T.I., Bradley, J., Lochner, J. and Iglewski, B.H. The Role of Exoenzyme S in *Pseudomonas aeruginosa* infections. J. Infect. Dis. 152:716-722, 1985.

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Foreword

       During the course of this work, the authors were greatly assisted by Mr.
Jack Lile, Mr. John Bradley, Ms. Lucia Davenport and Ms. Diane Unwin.   Their
help is deeply appreciated.   Portions of this research were done in
collaboration with Drs. J.C. Sadoff and A.S. Cross, Department of Bacterial
Diseases, WRAIR, Washington, D.C. 20012 and Dr. James Kenimer, Bureau of
Biologics (FDA), Washington, D.C. 20892.

       In conducting the research described in this report, the investigators
adhered to the "Guide for Laboratory Animals Facilities and Care" as
promulgated by the Committee on the Guide for Laboratory Animal Resources,
National Academy of Sciences, National Research Council.

       For the protection of human subjects the investigators have adhered to
policies of applicable Federal Law 45CFR46.

       The investigators have abided by the National Institutes of Health
Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and
the Administrative Practices Supplements.

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Figures

I. The Role of Exoenzyme S in *P. aeruginosa* Infections of Experimental Animals.

A. Studies in Burned Mice (see paper #1 appendix)

To define the contribution of exoenzyme S to the pathogenesis of infections with *Pseudomonas aeruginosa*, we compared the ability of an S⁻ mutant and an exoenzyme S⁺ producing parent, 388, to colonize and disseminate in burned mice. Both the exoenzyme S⁻ mutant and the S⁺ parent strain proliferated in burned skin, but only the parent strain was able to effectively disseminate to blood and other tissues. The possibility existed that the S⁻ mutant was somehow altered making it more susceptible to serum or killing by PMNs. We examined the LPS of the mutant, its serum sensitivity and its sensitivity to killing by PMNs and showed that it was identical to its parent strain in these respects. It was also equally motile as its parent strain. The alternative explanation for the inability of the S⁻ mutant to disseminate was that exoenzyme S alters the host defenses permitting dissemination. To test this hypothesis, we infected burned mice with equal mixtures of the S⁻ mutant and its S⁺ parent. Because of the presence of Tn1 in the mutant we were easily able to distinguish the S deficient mutant colonies from the parent strains by replicating on medium with carbenicillin. Examination of tissues and blood of these mice showed that the S⁻ mutant was able to disseminate in the presence of the S⁺ parent strain. These data suggested that exoenzyme S alters host defenses. This conclusion was strengthened by the observation that when mice were given S specific antibody (passively), dissemination of the S⁺ parent strain was greatly reduced (1). This study extended our previous ones showing that exoenzyme S contributes to virulence of *P. aeruginosa* in burned mice (2-4) as well as in chronic lung infection in rats (5).

B. Studies in Neutropenic Rats.

Studies on the contribution of exoenzyme S to virulence of *P. aeruginosa* were extended using a neutropenic rat model in collaboration with Dr. Alan Cross, Dr. Jerald Sadoff and Hugh Collins at WRAIR. Rats were made neutropenic by treatment with cytoxan. They were then inoculated with various numbers of either the S⁺ parent strain, (388), the S⁻ mutant, (388*exsl*::Tn1) or the S⁺ Tn1 induced leucine auxotroph. Two different routes of infection were used; oral and subcutaneous. When infected by the oral route, the S⁻ mutant was recovered from the stool but not from blood or other tissues. Further, none of these animals died. On the other hand, rats fed the S⁺ parent or leucine auxotroph, died within 12 days, even when given several logs fewer bacteria. These S⁺ organisms were recovered from the stool, t'   and other tissues. The bacteria recovered from these rats were tested for S phenotype, serotype, and carbenicillin resistance (where appropriate) and shown to be the same as that used in the inoculum. This ruled out the possibility that the dead rats had somehow been infected with a virulent organism from the environment. When the rats were infected with the S⁺ and S⁻ strain subcutaneously, the S⁻ mutant was several logs less virulent than the S⁺ strains. The S⁺ strains were recovered

from the skin (site of inoculation), blood, stool, and other tissues but the S˙ mutant was only recovered from the skin. These data indicated that exoenzyme S contributed to virulence of *P. aeruginosa* in neutropenic rats. Further, the data confirmed our previous conclusion (1) that exoenzyme S alters some aspect of the host defenses and suggested it is something other than the neutrophils. These studies should be extended using additional animals.

II. Quantitation of Human Antibody to Exoenzyme S by ELISA.

We developed an ELISA assay to quantitate S antibody. The methods used were adaptions of the procedures of Voller et al. (6). In preliminary experiments, we found that the 49,000 dalton (49Kd) form of exoenzyme S was better to use as the coating antigen as compared to the 53,000 dalton (53Kd) protein. We also found that the optimum concentration of antigen to use was 15ng/well. Following coating and washing, the plates are incubated with gelatin. In comparative experiments, we found gelatin superior to BSA in minimizing background reactivity. Following incubation with various dilutions of human serum and additional washes, goat anti-human immunoglobulin conjugated to horse radish peroxidase was added. Color was developed by adding ABTS, the reaction stopped with 50ul of 5% SDS after 30 min., and the absorbance determined with a Dynatech microelisa reader at 410nm.

We used the above ELISA to begin to quantitate S Antibody in human sera and to compare the ELISA titers to enzyme neutralization titers. Of the two assays, the ELISA was the more sensitive and reproducible, however there was generally a good correlation between the two assays; r = 0.9, see figure 1, p5.

For most of the bacteremic patients, we had only a few serum samples/patient. However, for several, we had numerous samples from throughout the infectious episode. This allowed us to follow the development of S antibody both by ELISA and enzyme neutralization. Figure 2,(p6) shows the chronological development of S antibodies in two such patients. In both patients, the first serum sample corresponded to the day of the first positive blood culture. As is seen, (Figure 2), both patients had no detectable antibody to S at the onset of their infection but both developed a vigorous immune response to exoenzyme S. Again there was good correlation between the ELISA titers and the enzyme neutralization titers. Clearly, exoenzyme S was made in these humans during *P. aeruginosa* infections as in toxin A (7), and they responsed to this antigen. To date, insufficient patients have been studied to draw any conclusions concerning the protective potential of S antibody; however, these studies should be expanded. This work was done in collaboration with Drs. Alan Cross and Jerald Sadoff of WRAIR.

III. Isolation of Exoenzyme S Specific Monoclonal Antibodies.

We isolated and partially characterized monoclonal antibodies specific for exoenzyme S. BALB/c mice were immunized with 10ug of purified 49Kd exoenzyme S protein in complete Freunds adjuvant injected IP on day 0. On days 7, 14 and 21 each mouse received 10ug of antigen, IP in incomplete Freunds adjuvant.

Animals were sacrificed 3 days later. Preliminary experiments indicated that this immunization protocol was optimal. Spleen cells were obtained and fused with SP/2 myeloma cells using the procedures described by Kenimer et al. (8). Fused cells were suspended in S/HAT medium and seeded in wells of microtiter plates (8). Media from wells with viable colonies were assayed for antibody by our ELISA (see II above). Selected hybridomas ware subcultured to determine stability and then subcloned by limiting dilution 2 times. From 4 separate fusions 8 stable clones were isolated which produced monoclonal antibody to exoenzyme S. Two of these monoclonal antibodies were IgMs and 6 were IgGs. All 8 monoclonals reacted well with the 49Kd form of exoenzyme S on western blot as well as in ELISA. Five of these monoclonals neutralized S enzymatic activity greater than 60% whereas 3 had either very low or no neutralizing activity. These clones were expanded and stored in liquid nitrogen. This work was done while I was on sabbatical leave at FDA in collaboration with Dr. James Kenimer.

IV. Cloning Exoenzyme S Genes.

Preliminary data indicated that cosmid clones were present in our DNA bank which complemented the 388 _exsl_::Tnl mutation. Since plasmid DNA was not recoverable from the 388 _exsl_::Tnl host we began to search for the HB101 host containing S recombinant sequences by a batch mating protocol. _E. coli_ HB101 pLAFR-388 cosmid clones were plated on selective medium and individual colonies were picked into microtiter trays. Ninety six colonies per mating were replicated onto selective medium and used as donors in a triparental cross in 388 _exsl_::Tnl. S preduction by 388 _exsl_::Tnl exconjugants was determined by the colony blot assay utilizing antisera specific for the 49Kd active form of exoenzyme S. A total of thirteen matings were performed (1248 colonies) before we detected a microtiter plate which contained a presumptive positive clone. The plate was divided into smaller sections of donor colonies until the single HB101 containing the S recombinant cosmid clone was found (pDF100).

Preliminary analysis indicated that pDF100 complemented the mutation in 388 _exsl_::Tnl cells. We confirmed this repeatedly. ADP-ribosyltransferase activity was found in culture supernatants of 388 _exsl_::Tnl containing pDF100 but not in the mutant that contained only the pLAFR vector. Western blot profiles of 388 _exsl_::Tnl pDF100 and the S$^+$ parent 388 pLAFR using concentrated culture supernatants as antigen and anti-49Kd antibody were indistinguishable. In contrast 388 _exsl_::Tnl (S$^-$) was negative. Restriction mapping analysis indicated that pDF100 contained approximately 28kb of _Pseudomonas aeruginosa_ chromosomal DNA. EcoRI digestion of pDF100 resulted in fou pieces of DNA that contained 11kb, 8.2kb, 5kb and 3.8kb. The EcoRI fragments from pDF100 were subcloned into pLAFR (pDF101, pDF102, pDF103 and pDF104), and tested for activity by mating these plasmids into 388 _exsl_::Tnl. One subclone, pDF102, contained the 8.2kb EcoRI insert from pDF100 and it demonstrated reactivity with anti-49Kd antibody on colony blots. A partial restriction map was constructed of pDF102 (see Figure 3, p7). In order to localize the gene for exoenzyme S we mutagenized pDF102 with the transposon Tn501 (9). Transposon mutants were prepared by transforming _E. coli_ HB101 that contained our target

plasmid, pDF102, with RSF1010::Tn501. Lambda CI857 lysates were prepared from strains that exhibited both tetracycline and mercury resistant phenotypes. The lysates were used to transduce recombinant plasmids to *E. coli* HB101 hosts. Transductants that contained both mercury and tetracycline resistance markers were passaged to insure stability and introduced into 388 *exs1*::Tn1 by conjugation and tested for exoenzyme S positive production. Eleven exoenzyme S negative and two exoenzyme S positive Tn501 insertions were isolated. Restriction mapping analysis and Southern blot probing located putative exoenzyme S sequences to a 3kb region on one side of the 8.2kb EcoRI insertion in pDF102 (see Figure 3, page 8). These studies should permit one to subclone the S gene on a piece of DNA approximately 3kb in size, characterize it, and construct isogenic S^+, S^- mutants in virtually any strain of *P. aeruginosa*.

V.   Amino Acid Sequence Analysis of Exoenzyme S.

We determined the amino acid sequence, starting at the amino terminus, of the 49Kd exoenzyme S protein. Three separate preparations of the 49Kd exoenzyme S protein were analyzed; each two separate times. In each case, a total of 38 to 40 cycles were obtained on the sequenator. There was perfect agreement for each run on all three preparations for the first 22 amino acids but considerable discrepancy beyond amino acid 22. The sequence of the first 22 amino acids of the 49kd S protein was, Met-His-Ile-Gln-Ser-Leu-Gln-Gln-Ser-Pro-Ser-Phe-Ala-Val-Glu-Leu-His-Gln-Ala-Ala-Ser-Gly X. We also attempted to sequence the amino terminal portion of the 53Kd exoenzyme protein. We analyzed four separately purified preparations of the 53Kd protein. However, we were unable to obtain any sequence data. Apparently the amino terminus of our purified 53Kd S protein was blocked. The sequence for the first 22 amino acids of the 49Kd S protein should, however, be useful in designing synthetic oligonucleotide probes to determine if the S gene we cloned is the structural gene encoding the 49Kd exoenzyme S protein and for screening *Pseudomonas aeruginosa* DNA banks to identify clones containing the S structural gene.

Figure 1. Correlation of ELISA reactivity and enzyme neutralization titers. The peak serum ELISA reactivity of each patient is compared to the inverse log2 of the dilution of the same serum sample necessary to achieve 50% enzyme neutralization. The plot yielded a correlation co-efficient of r = 0.9.
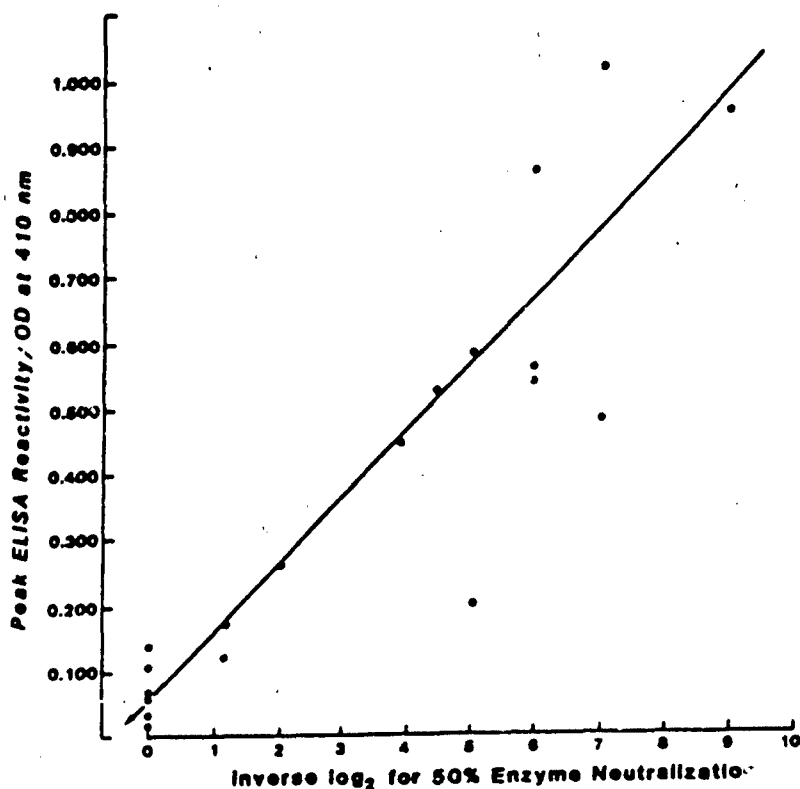
Figure 2. Chronological development of antibody to exoenzyme S in the
sera of two patients with P. aeruginosa bacteremia.. Circles connected
with dotted lines represent enzyme neutralization titers whereas circles
connected with solid lines represent ELISA titers. Open circles
represent patient #1 and the closed circles patient #2.
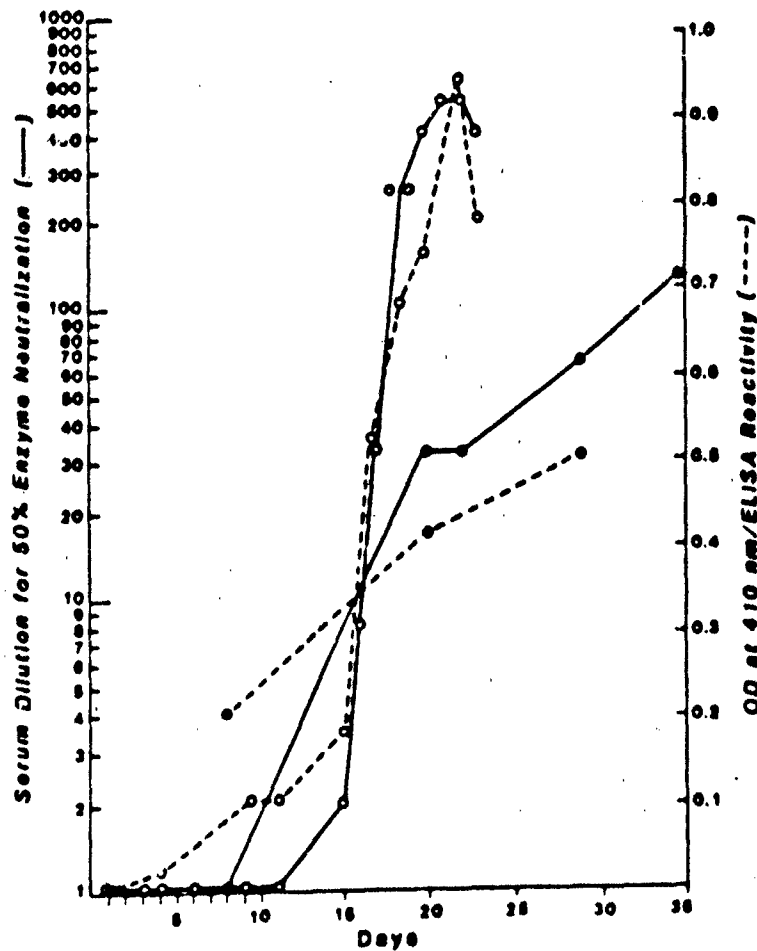
Figure 3. Partial restriction map of P. aeruginosa 388 DNA in pDF102.
Restriction endonuclease sites for EcoRI (E), Sal I (S), and XHO I (X)
are shown. The locations of Tn501 insertions (⚲) were determined by
restriction analysis and Southern hybridization. When Tn501 containing
plasmids were mated into the S⁻ mutant 388 exs1::Tn1, numbers 1 and 13
demonstrated S activity. Plasmids corresponding to numbers 2-12 were S
negative.

pDF 102

1 Kb